

Spring 5-1-2018

Composition and Function of the Bacterial Consortium Associated with the Accessory Nidamental Gland of the Hawaiian Bobtail Squid

Jessica Bertenshaw
jessica.bertenshaw@uconn.edu

Follow this and additional works at: https://opencommons.uconn.edu/srhonors_theses



Part of the [Environmental Microbiology and Microbial Ecology Commons](#)

Recommended Citation

Bertenshaw, Jessica, "Composition and Function of the Bacterial Consortium Associated with the Accessory Nidamental Gland of the Hawaiian Bobtail Squid" (2018). *Honors Scholar Theses*. 574.
https://opencommons.uconn.edu/srhonors_theses/574

Composition and Function of the Bacterial Consortium Associated with the Accessory
Nidamental Gland of the Hawaiian Bobtail Squid

Jessica Bertenshaw

Spencer V. Nyholm, Ph.D.

Associate Professor

Department of Molecular and Cell Biology

University of Connecticut

Honors Thesis Department of Molecular and Cell Biology

December 8, 2017

Table of Contents

Abstract	2
Introduction	3
Materials and Methods	
Identification of Isolates	5
Antibiotic Effects	7
Defense Against Algae	8
Results	9
Discussion	16
Conclusions	19
Acknowledgements	19
Literature Cited	19

Abstract

The bacterial consortium associated with the accessory nidamental gland (ANG) of the Hawaiian bobtail squid, *Euprymna scolopes* is posited to confer symbiotic benefits to the eggs of the squid when bacteria are transferred from the gland of the mother to the jelly coat, which surrounds the embryo in the egg. To characterize the composition of this community bacterial isolates from the egg jelly coat and from the ANG were identified using 16S sequencing. To elucidate the function of these bacteria, egg development experiments were performed to determine the effect of antibiotics on egg survival and the

composition of the bacterial community. Experiments were also performed to test the potential effect of the bacteria associated with the eggs on algal biofouling.

Introduction

Many animals that lay their eggs in aquatic environments must defend their eggs from biofouling and disease. The broad field of bacterial symbioses encompasses a multitude of host-microbe interactions that play an important role in the defense of these animals' eggs. One of these animals is the Hawaiian bobtail squid, *Euprymna scolopes*. This squid is native to the bays of the islands of Hawaii. These animals are nocturnal and in order to hunt at night they need camouflage against the moonlight that would reveal their location to predators by showing their silhouette against the moon. The squid have an organ known as the light organ, which houses the bacterium *Vibrio fischeri*. *V. fischeri* are bioluminescent and when they glow in the light organ of the squid they provide the camouflage that the host requires (Jones & Nishiguchi 2004).

The Hawaiian bobtail squid serves as a model for beneficial host-microbe interactions, quorum sensing, and host specificity (McFall-Ngai 2014, Nyholm and Graf 2012) but is also studied for the bacterial symbiosis associated with egg defense in the female squid. The lesser-studied bacterial symbiosis associated with *E. scolopes* is potentially equally important for the survival of the species. The eggs of the Hawaiian bobtail squid are laid in groups of 50-200 eggs known as a clutch on coral rubble where they are left to develop for up to a month before hatching without any parental care (Singley 1983). These eggs are largely reported to avoid biofouling from algae and fungus in both *E. scolopes* and in many other cephalopods that also have an accessory

nidamental gland (ANG) including the myopsids, sepiids, and sepiolids (Biggs and Epel 1991; Buchner 1965; Fields 1965). The ANG is part of the reproductive system of the female squid and contains Gram-negative bacterial symbionts from the *Alphaproteobacteria*, *Opitutae*, *Flavobacteriia* and *Gammaproteobacteria* classes (Collins et al. 2012). The Nyholm lab has 97 isolates from the accessory nidamental gland and jelly coat of the eggs in culture. Many of these strains had been previously identified and with the new identification of 44 isolates by 16S sequencing there are a total of 95 identified isolates in culture. The ANG deposits these bacteria into the jelly coat (JC), a viscous material secreted by the nidamental gland which coats each egg yolk sac and developing embryo (Collins et al. 2012). The squid egg is then enclosed in an outer capsule. The bacteria incorporated into the JC from the ANG are posited to confer symbiotic benefits to the developing embryo through the production of secondary metabolites. The incorporation of bacteria into the egg JCs was first hypothesized to serve a protective function in the early 1990s (Biggs and Epel 1991), but until recently this hypothesis remained untested and the exact mechanism by which this could be accomplished was unknown. Recent research from Nyholm and Balunas labs at UConn has demonstrated that bacterial extracts from certain ANG/JC isolates inhibit other bacteria (Gromek *et al.* 2016). Other research has demonstrated that *E. scolopes* eggs resist the growth of the fungus *Fusarium keratoplasticum*. When treated with antibiotics these eggs developed a dense fungal biofilm, which overgrows the clutch and causes death of the embryos (Kerwin *et al.*, in prep.). Experiments done with parts of the eggs as opposed to whole eggs have shown that the jelly coat and its bacterial symbionts are the integral component in fungal resistance (Kerwin *et al.*, in prep.). Egg development

experiments were performed to demonstrate the effects of the antibiotics on the development of the eggs as well as the effects on the bacterial consortium. Marine algae are capable of biofouling (Mieszkin 2013), and may also present a risk to the developing *E. scolopes* eggs. Egg development experiments as well as co-culture experiments were performed in order to determine the possible defensive properties the bacteria could have against algae. The overall goal of the experiments performed is to determine what role the bacterial symbionts play in defending the eggs against possible biofouling agents.

Materials and Methods

Identification of Isolates

Isolate Culturing: Bacterial colonies were isolated from either the tissue of the accessory nidamental gland (ANG) of the Hawaiian bobtail squid or the jelly coat of the eggs.

These isolates were identified to help characterize the community found in the ANG of the squid. For isolation, the tissue of the ANG or the jelly coat were dissected and homogenized in filter sterilized squid ringers. The homogenate was then plated onto seawater tryptone (SWT) agar (Gromek *et al.* 2016) and incubated at 27°C for 3 days. The colony morphology was noted for each isolate and images were obtained of the colonies (Appendix A).

16S Amplification Analysis: Polymerase chain reaction (PCR) mixtures were prepared using a toothpick dipped into a bacterial colony on the SWT plate to introduce the bacterial DNA to the sample. The standard PCR reaction mixture contained 16S primers for 27F and 1492R. The PCR was run on the Bio-Rad iCycler thermal cycler with a denaturation step of three minutes at 95°C, an annealing step of 30 seconds at 55°C and

an extension step of a minute and 30 seconds at 72°C. The PCR product was then treated using the ExoSAP-IT PCR product cleanup kit by Thermofisher. A BigDye sequencing PCR reaction was performed on the sample. This reaction mixture contained 0.75ul of BigDye reagent, 0.8 ul of either the 27F primer or the 1492R primer, 2.45 ul of molecular grade water, and 1 ul of the cleaned PCR product. This was run on the thermal cycler and then a DNA precipitation was performed. For the DNA precipitation 1.25 ul of EDTA, 15 ul of 99% ethanol, and 5 ul of the BigDye product were combined and allowed to incubate at room temperature for 15 minutes. The tubes were then centrifuged at 20,817xG at 4°C for 10 minutes. The supernatant was discarded and the pellet was re-dissolved in 125 ul of 70% ethanol. The sample was centrifuged again at the same temperature and speed. The supernatant was again removed and the sample tubes were placed open on a heat block at 95°C for 5 minutes. The sample was then sent to the Center for Genome Innovation (CGI) for processing. The resulting sequences were analyzed using BLAST and RDP databases to identify taxa. Images were also taken of all the isolates grown on SWT plates from the cultures (Appendix A).

Conversion Factors: Growth conversion factors for certain isolates were also determined so that these isolates could be used in other experiments. Conversion factors allow the optical density reading at a wavelength of 600 nm (OD600) of a liquid culture of the isolate to be equated to colony forming units per milliliter. These were determined by growing cultures of isolates in SWT broth and then plating dilutions of these cultures onto SWT plates. The liquid cultures consisted of 3 mL of SWT broth inoculated with colonies of the bacteria grown on SWT plates. These liquid cultures were incubated on shaking racks at 30°C for approximately 3 hours. The shaking allowed for proper

oxygenation of the cultures. The colony forming units were counted for each plate at a given dilution so that the conversion factors could be calculated (Table 1).

Antibiotic Effects

Experiments were performed to determine the effect that antibiotics have on the bacterial consortium in *E. scolopes* eggs. Previous experiments had used either an antibiotic cocktail containing penicillin G, kanamycin, spectinomycin, streptomycin, and gentamicin at a final concentration of 25 ug/mL or a solution of chloramphenicol also at 25 ug/mL. In these experiments, eggs were treated with the antibiotics and challenged with the fungus *Fusarium keratoplasticum* (Kerwin *et al.*, in prep.). Eggs that had been treated with the antibiotics when compared to control eggs exposed to fungus but not treated with the antibiotics developed larger biofilms of the fungus and the embryos in the treated group did not develop as well (Kerwin *et al.*, in prep.). These experiments assume that the antibiotics used were affecting all members of the bacterial community equally in the eggs. Further experiments were conducted without fungus to see how the bacterial community was changed by treatment with antibiotics to confirm this assumption. Trials were run where eggs were placed in well plates and allowed to develop for either 10 or 18 days. Water changes were performed every other day and at each water change new antibiotics were added to the treatment wells. The experiments were often contaminated with fungus from unknown environmental sources (Figure 3). At day 0, 10, and 18 the jelly coats were dissected from five eggs and homogenized in 500 uL of filter sterilized squid ringers. This solution was then plated at various dilutions. After three days the plates were counted (Figure 4). Bacterial isolates were also isolated

and identified from these plates. These identifications can be found in Appendix A with labels CB 14-33.

Defense Against Algae

Egg Development: I hypothesized that the bacteria in the eggs of the Hawaiian bobtail squid might have protective effects against potentially harmful algae. In order to test this hypothesis, two types of experiments were performed. In the first set of experiments, individual eggs were separated from a clutch and each placed in an individual well of a 12 well plate (Figure 6). Half of the eggs were treated with an antibiotic cocktail to remove the bacteria from the jelly coat and the other half were not. Also half of the eggs (half of the treated eggs and half of the untreated eggs) were exposed to a lower concentration of algae while the other half were exposed to a higher concentration of algae. For the trial with *Isochrysis galbana* the lower concentration of algae was 1×10^3 algae cells/mL and the higher concentration was 1×10^4 algae cells/mL. For the *Bangia fuscopurpurea* the cells were unable to be visualized on the hemocytometer so exact concentrations could not be determined. Instead either 0.5 mL for the lower concentration or 1 mL for the higher concentration of the stock culture of algae was added to make a 3 mL final volume in each well of the plate. The egg development was tracked over a 30-day period where new algae and antibiotic cocktail were added when a water change was performed every other day. Due to the small volume of filter-sterilized seawater that the eggs were in, frequent water changes and the use of a rocker were necessary to keep the water oxygenated. The first set of experiments was performed with the alga *Isochrysis galbana* (Figure 5A). This alga was chosen for its ability to be grown easily and reliably

in a laboratory environment and because it naturally inhabits a marine environment. It also prefers similar light and temperature specifications to the eggs of the squid. Experiments were also run with another type of algae called *Bangia fuscopurpurea* (Figure 5B). This alga was chosen for similar reasons to the *Isochrysis galbana*. It also is a red alga instead of a green alga to add diversity to the types of algae tested.

Co-culture: Co-culture experiments were also performed. *Isochrysis galbana* at a concentration of either 1×10^2 or 1×10^3 algal spores/mL were combined with bacteria isolated from the jelly coat of the eggs, named JC-28, at a concentration of 1×10^6 CFU/mL for a total of 3 mL in each test tube. The bacteria and algae were combined in different ratios, either 1:1 or 1:5 (bacteria: algae) The test tubes were put on a rocker to shake in the same light and temperature conditions as the algae cultures for 18 hours. At 18 hours the samples were examined with the hemocytometer to get algal cell counts and the samples were also plated onto SWT plates to determine bacterial cell counts. The bacteria were plated at 1×10^{-5} and 1×10^{-6} dilutions. Conversion factors were used to convert the raw counts to concentrations of bacteria and algae. In order to obtain the conversion factor for the *Isochrysis galbana* alga an absorbance curve was created following the protocol previously described (Figure 9, Kaplan et al., 1985).

Results

Identification of Isolates

A total of 21 different genera were found with a total of 51 different isolates identified (Figure 2). These were distributed across four classes (Figure 1). The class with the most

isolates was the Alphaproteobacteria. Within this class the *Phaeobacter* and *Ruegeria* were the most abundant genera.

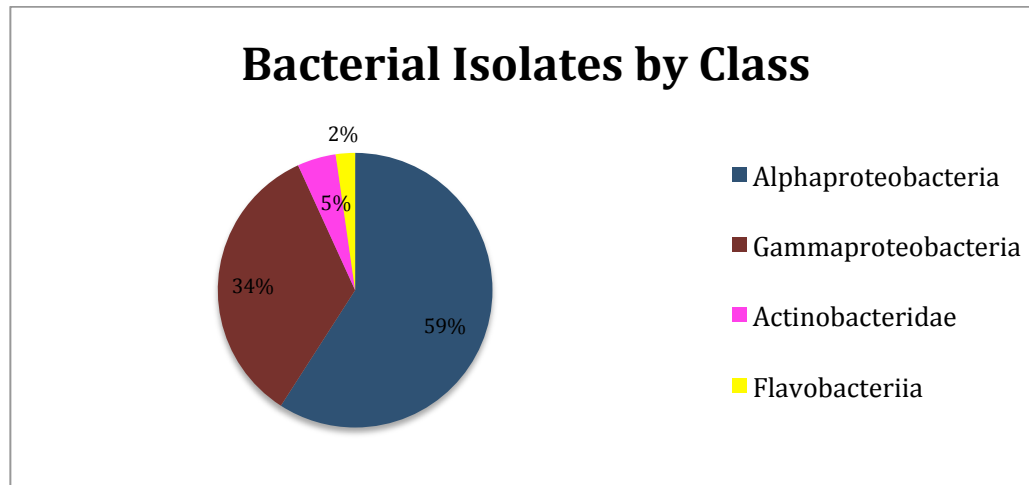


Figure 1: Bacterial isolates from ANG and jelly coat samples grouped by class.

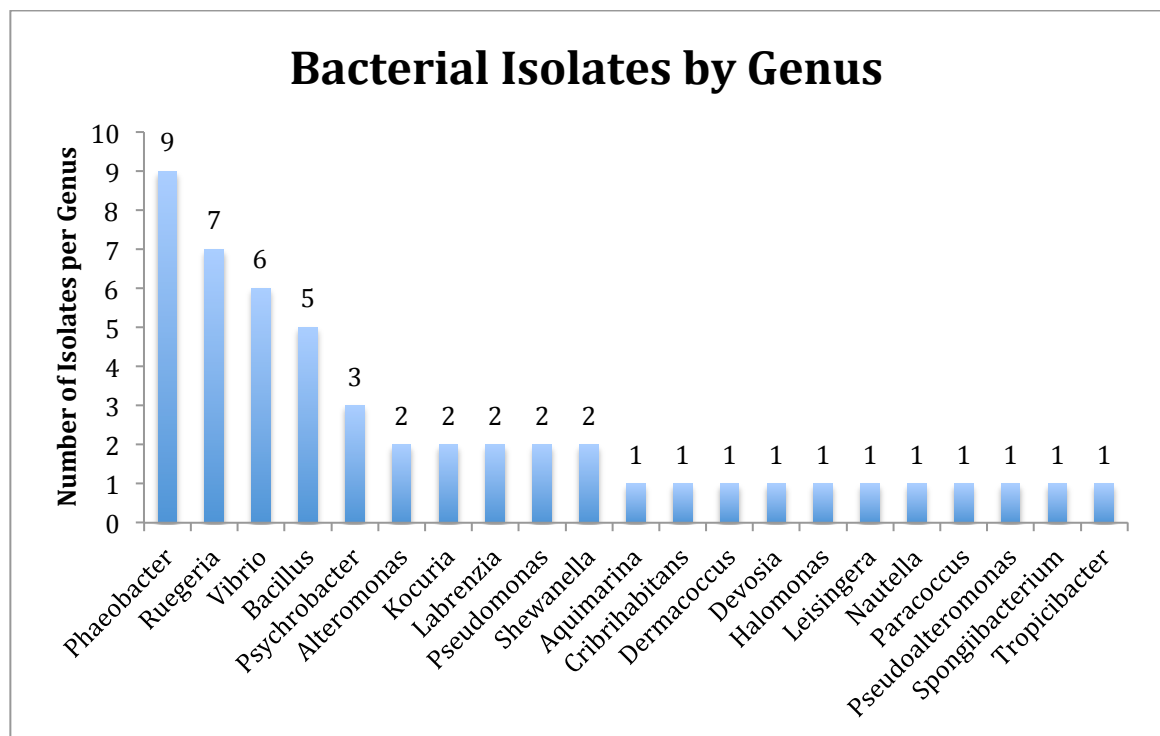


Figure 2: Bacterial isolates from ANG and jelly coat samples grouped by genus.

Conversion Factors: Numerical factors that allow for the conversion between absorbance values obtained from the spectrophotometer and CFU/mL concentrations were obtained for four isolates from the jelly coat (JC) and ANG (Table 1). These conversion factors allowed for use of these isolates in other experiments.

Isolate	Conversion Factor (CFU/mL)/OD600
<i>Pseudoalteromonas sp.</i> JC-34	1.1×10^8
<i>Leisingera sp.</i> ANG-7	1.1×10^8
<i>Leisingera sp.</i> ANG-59	2.4×10^8
<i>Nautella sp.</i> M5	6.5×10^8

Table 1: Conversion factors for various ANG and JC isolates for conversion between plate counts and optical density readings at 600 nm.

Antibiotic Effects: Many of the eggs treated with antibiotics in trials run were contaminated with fungus from environmental sources (Figure 3). In trials where data could be obtained without contamination, treatment with the antibiotic cocktail and chloramphenicol initially lowered the number of bacteria in the jelly coat. By day 19 however, the number of bacteria were observed to increase beyond even the original counts from day zero. The eggs that did not receive treatment had a consistent number of bacteria across the course of the experiment (Figure 4).

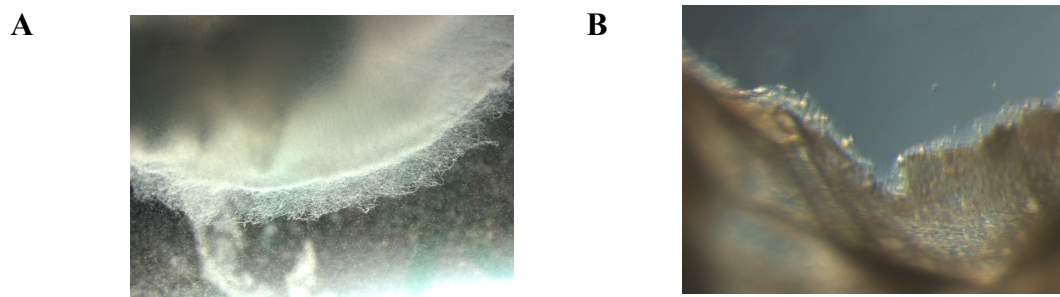


Figure 3: Image of whole egg treated with antibiotic cocktail (A) and whole egg not treated with the antibiotic cocktail (B), taken on the Discovery V20 stereoscope. Fungus seen on image A is from environmental sources.

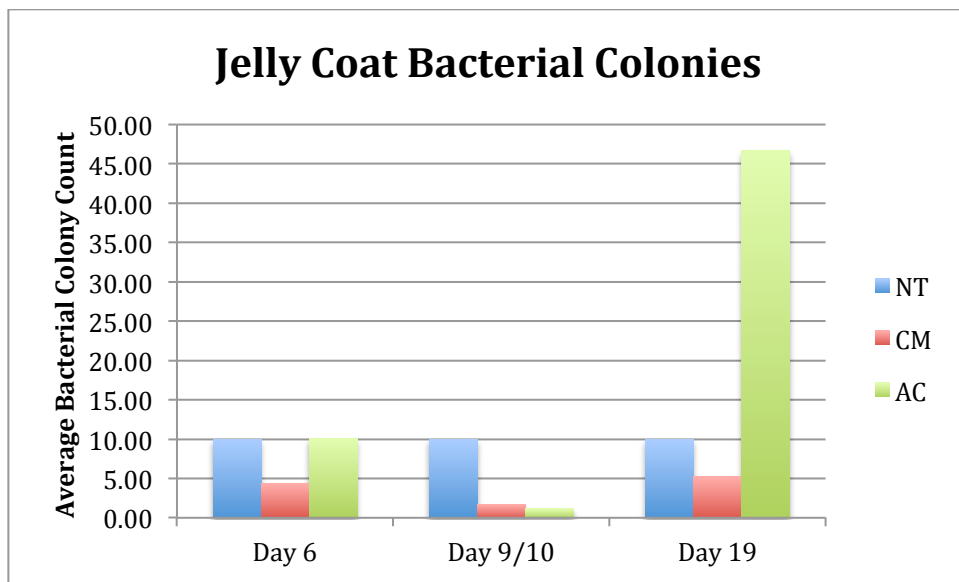


Figure 4: Average number of bacterial colonies counted on plates after one day of incubation after plating of jelly coat homogenate at 5, 10, or 18 days of egg development, eggs either received no treatment (NT), chloramphenicol treatment (CM), or antibiotic cocktail treatment (AC).

Defense Against Algae

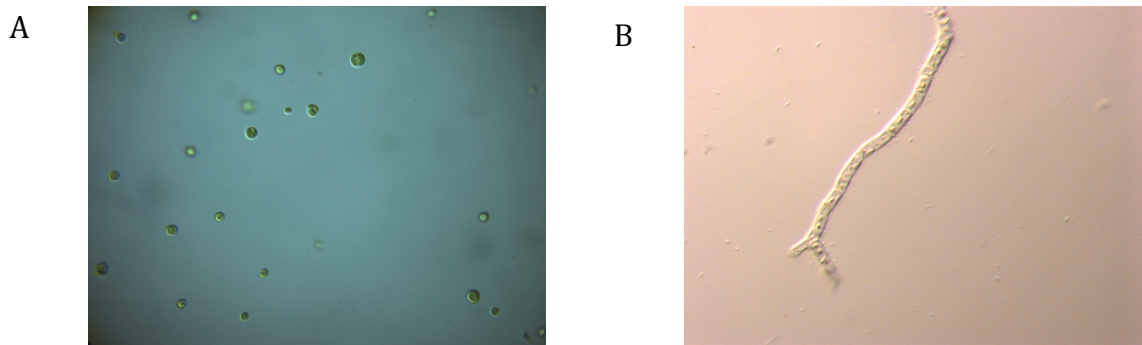


Figure 5: *Isochrysis galbana* (A) and *Bangia fuscopurpurea* (B) cultures visualized with a Zeiss axiovert microscope (A) and Discovery V20 stereoscope (B), both algae are marine algae, *Isochrysis galbana* is a green alga, *Bangia fuscopurpurea* is a red alga.

Egg Development: Eggs were exposed to various conditions and their development was observed (Figure 6). These experiments were performed with both *Isochrysis galbana* and *Bangia fuscopurpurea* (Figure 5). At day 18 of the *Bangia fuscopurpurea* egg development trial there was more algae on the eggs being treated with antibiotics as

compared to those not being treated. However, overall there was not much observable difference between the appearance of algae on the treated and untreated eggs for both types of algae used (Figure 7). In the *Isochrysis galbana* experiments, the eggs at the lower concentration hatched but the eggs at the higher concentration did not hatch suggesting that the algae did have an affect on the development of the eggs.

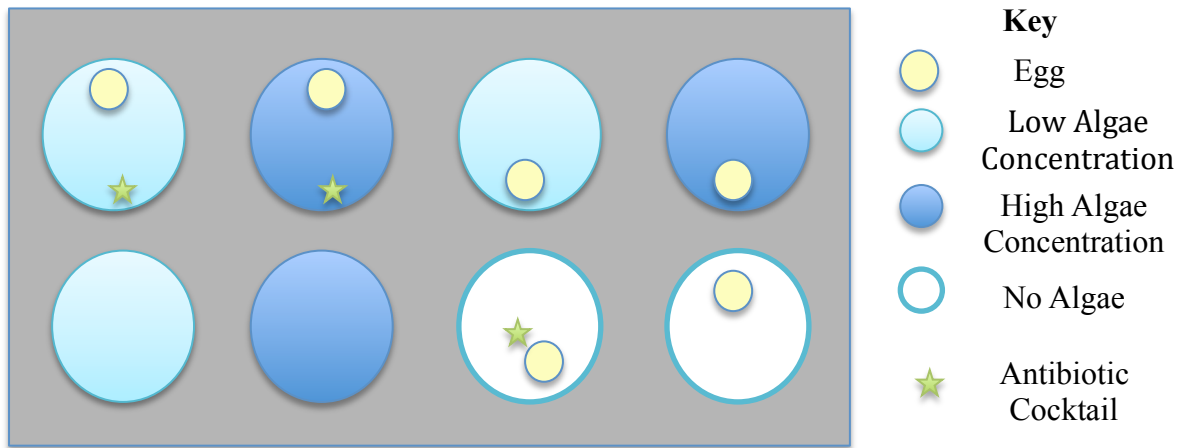


Figure 6: Egg development experiment set up, 12 well plate with eggs in various treatment conditions, in filter sterilized sea water, on a rocker to increase aeration, n=2

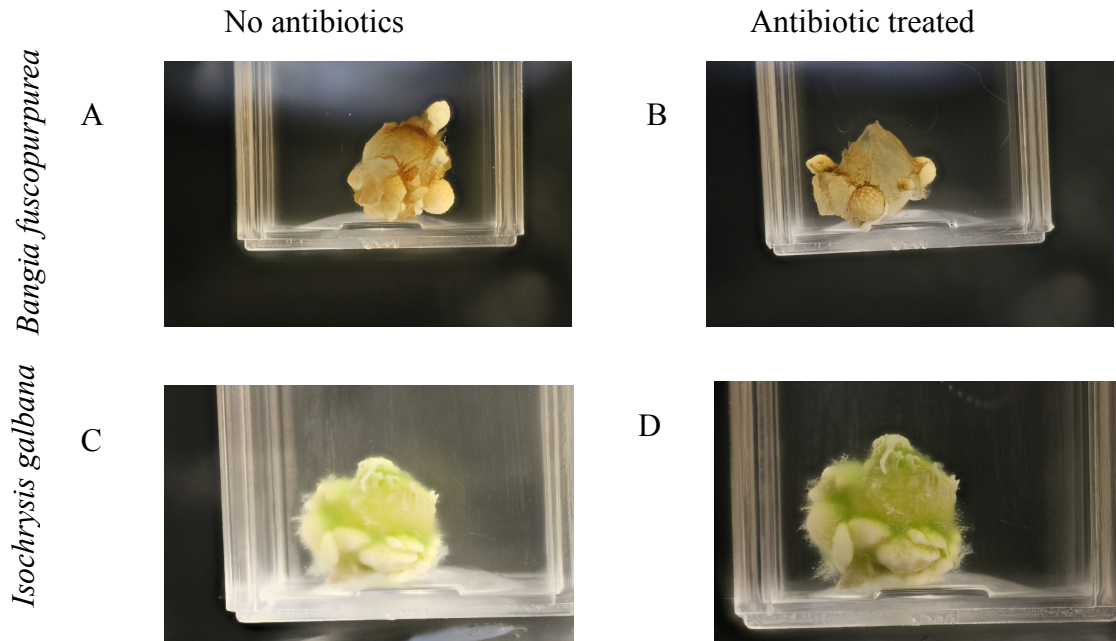
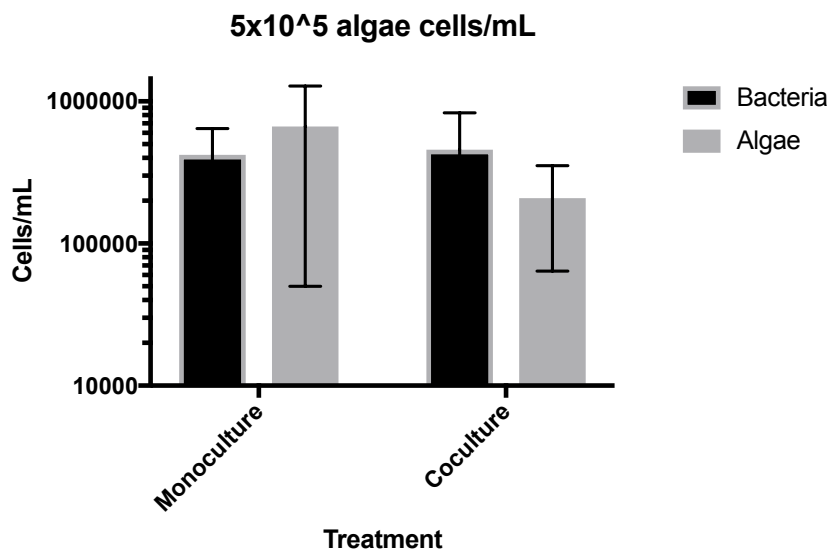


Figure 7: Eggs treated with antibiotics (B,D) or not (A,C) and exposed to either *Isochrysis galbana* (C,D) or *Bangia fuscopurpurea* (A,B), *Isochrysis galbana* was added at a concentration of 1×10^4 algal cells/mL on eggs pictured.

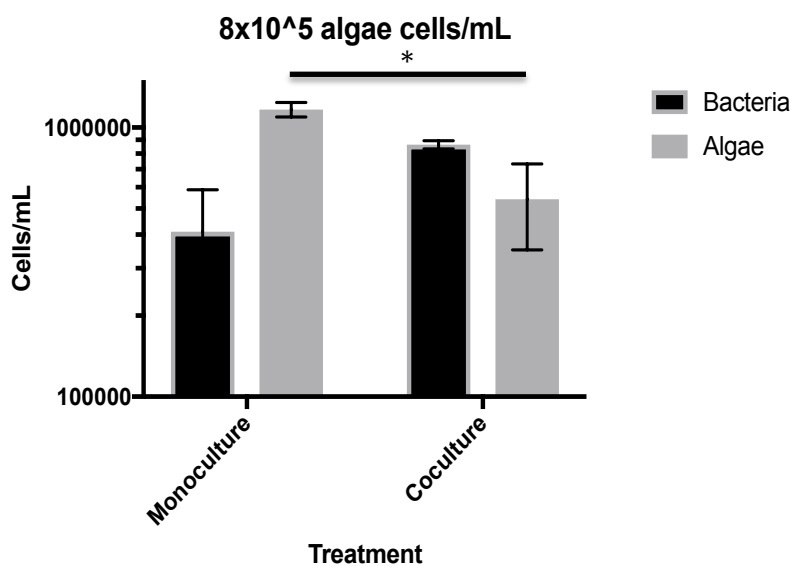
Co-culture: Fewer algae were observed in co-culture experiments when they were combined with JC-28 than compared to when cultured alone (Table 2). There is a statistically significant decrease between the algae at 8×10^5 algae cells/mL in the monoculture control tube and the 1:5 (JC-28: algae) co-culture tube with a p value of 0.0001 (Figure 8). An absorbance curve was also created in order to determine the wavelength at which to measure the algal culture for peak absorbance. The absorbance values were also used to create a conversion factor to calculate the algal cell/mL concentration from a spectrophotometer absorbance reading (Figure 9). The peak absorbance was determined to be 700 nm.

Table 2. Co-culture experiment results after an 18-h incubation performed with *Isochrysis galbana* and JC-28. All tubes had a final volume of 3 mL. (n=3)

Tube Condition	Algae (cells/mL)	JC-28 (CFU/mL)
Algae alone (5×10^5 algae cells/mL)	1.0×10^6	0
Algae alone (8×10^5 algae cells/mL)	1.1×10^6	0
Bacteria alone (5×10^5 CFU/mL)	0	3.6×10^5
Bacteria alone (2×10^5 CFU/mL)	0	3.6×10^5
1:1 ratio (JC-28: algae)	1.9×10^5	3.5×10^5
1:5 ratio (JC-28: algae)	5.9×10^5	8.7×10^5



Source	DF	MS	F-ratio (DFn,DFd)	P Value
Bacteria vs Algae	1	1.84E+11	F (1, 8) = 1.242	P=0.2975
Monoculture vs Coculture	1	1.33E+11	F (1,8) = 0.8985	P=0.3709
Interaction	1	3000000	F (1, 8) = 2.024e-005	P=0.9965
Residual	8	1.48E+11		



Source	DF	MS	F-ratio (DFn, DFd)	P Value
Bacteria vs Algae	1	1.42E+11	F (1, 8) = 7.651	P=0.0244
Monoculture vs Coculture	1	2.21E+10	F (1, 8) = 1.191	P=0.3068
Interaction	1	8.72E+11	F (1, 8) = 47.01	P=0.0001
Residual	8	1.86E+10		

Figure 8: Data from Table 2 with 2-way ANOVA test for statistical significance, 5×10^5 algal cells/mL represent 1.5 mL of algae (1:1 ratio), 8×10^5 algal cells/mL represent 2.4 mL of algae (1:5 ratio), monoculture represent control tubes with either bacteria or algae, co-culture tubes contained both algae and bacteria, statistically significant values are bolded, in the 1:5 ratio co-culture tubes the decrease in algal cells after 18 hours of incubation is statistically significant.

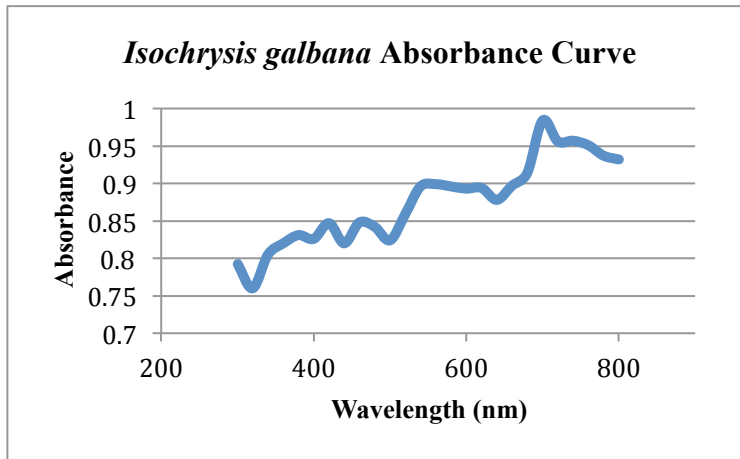


Figure 9: Absorbance curve of *Isochrysis galbana*, plotted absorbances over a range of wavelengths to determine the peak absorbance (700 nm)

Discussion

Identification of Isolates

The identified isolates fell into four distinct classes (Figure 1). A total of 21 different genera were found with a total of 51 different isolates identified (Figure 2). The PCR primers used in these experiments are specific to the highly conserved 16S region of the bacterial genome. Within this region there are variable regions, which allow for specific identification down to the genus level; the species cannot be determined with confidence in all cases. The most abundant class found in these samples was the *Alphaproteobacteria* (Figure 1). In previous work using culture independent methods, the most abundant class was also found to be the *Alphaproteobacteria* (Collins et al., 2012; Kerwin and Nyholm, 2017). The second most abundant class found was the *Gammaproteobacteria* (Figure 1).

In experiments using the culture independent method the *Gammaproteobacteria* were represented in much lower numbers (Kerwin and Nyholm, 2017). This indicates that the culture methods used in these experiments were particularly conducive to the growth of *Gammaproteobacteria*. The ocean is an environment with many microbes and with the eggs left to develop without parental care they are in need of defense from possibly harmful microbes. Identifying bacteria that could possibly offer these defenses to not just the eggs of *E. scolopes* but to the eggs of other marine organisms could expand the understanding of egg defenses and host-microbe-microbe interactions. Future experiments should include identifying more isolates from the ANG and JC of the squid to have a more complete picture of the bacterial community.

Antibiotic Effects

While the bacterial count numbers varied between trials and did not show a consistent decrease between the control and treatment eggs, there was a decrease in the number of different types of colonies observed on the plates at day 19 indicating a change in the overall community composition. In the eggs treated with antibiotics, there was less diversity observed in the colonies that grew on the plates. This could indicate that the reason the number of colonies increased so much by the final time point was due to selection for antibiotic resistant bacteria. Once other strains of bacteria were killed off earlier in the experiment these strains may have been able to grow to much higher densities without competition. In general it was difficult to obtain accurate counts due to weed-like colonies and the fungal contaminants. However, when these experiments were later repeated, it was observed that the antibiotics do decrease the bacterial population in

the jelly coat of the eggs as hypothesized (Kerwin *et al.*, in prep.). This indicates that experiments that use the antibiotics under this principle are valid because they do perform in the expected manner. The differences in these two experiments may be attributed to different levels of putative antibiotic resistant bacterial strains in the different sets of eggs tested.

Defense Against Algae

The development experiments showed no apparent difference in algae growth on the eggs between the two treatment conditions based on images taken with a dissecting microscope (Figure 7). At the end of the experiment with *I. galbana* all of the eggs from the lower algae concentration hatched while the eggs in the higher algae concentrations did not ever hatch. At day 18 of the *B. fuscopurpurea* trial there appeared to be more algae on the eggs treated with the antibiotic cocktail than the untreated eggs. However, for the rest of the experiment the amount of algae on the eggs appeared to be the same in both treatment groups. In future experiments, better quantitative techniques should be developed to accurately compare fouling algae under these conditions. In the co-culture experiments, there was a statistically significant decrease in the number of algal cells/mL in tubes co-incubated with JC-28 as compared to those tubes without the bacteria (Table 2, Figure 8). This is significant because it demonstrates that a bacterium associated with the eggs of the Hawaiian bobtail squid may have anti-algal effects. It is known that bacteria and algae can interact and affect the growth of one another (Mieszkin et al., 2013). Since eggs are in need of defense when they are left to develop in the ocean where there are many microorganisms in the water including possibly pathogenic algae it could

be beneficial for the bacterial consortium to play this role. In the future these experiments should be repeated to have a more robust data set. Different types of algae should be tested as well as different strains of bacteria isolated from the squid to see if the ANG/JC bacteria have broad antimicrobial activity.

Conclusions

These experiments have significance in the bigger picture of symbioses as well as for research into anti-algal products. The identification of the members of the bacterial consortium associated with the reproductive tract of the Hawaiian bobtail squid shed light onto the relationship between the squid and their symbionts. Characterization of this symbiotic community will allow for comparisons to other bacterial symbioses.

Verification of the methods used with the antibiotic cocktail experiments will help to verify other experimental findings to better understand the functional role of the ANG/JC symbiosis. Furthermore, the algal experiments demonstrate that strains of bacteria could possibly serve as anti-algal agents. Overall, this research increases our knowledge about the defensive properties and function of this bacterial consortium.

Acknowledgements

Thanks to Dr. Spencer Nyholm for serving as a mentor and thesis advisor. Thanks also to Dr. Allison Kerwin, Andrea Suria, and Sarah McAnulty for teaching me, helping guide my experiments, and allowing me to assist in their work in the lab.

Literature Cited

- Anderson, R. 2005. *Algal Culturing Techniques*. Academic Press. Chapter 3, Marine Culture Media.
- Biggs, J., Epel, D. 1991. Egg capsule sheath of *Loligo opalescens* Berry: Structure and association with bacteria. *JEZ*. **259**.
- Buchner, P. 1965. *Endosymbiosis of Animals with Plant Microorganisms*. New York: John Wiley & Sons, Inc. Chapter 9, Symbiosis in Luminous Animals.
- Collins, A., LaBarre, B., Wong Won, B., Shah, M., Heng, S., Choudhury, M., Haydar, S., Santiago, J., Nyholm, S. 2012. Diversity and Partitioning of Bacterial Populations within the Accessory Nidamental Gland of the Squid *Euprymna scolopes*. *Applied and Environmental Microbiology*. **78**.
- Fields, W.G. 1965. The structure, development, food relations, reproduction, and life history of the squid *Loligo opalescens* Berry. *Fish Bulletin*. **131**: 1–66.
- Biggs, J., Epel, D. 1991. Egg capsule sheath of *Loligo opalescens* Berry: Structure and association with bacteria. *JEZ*. **259**.
- Goecke, F., Labes, A., Wiese, J., Imhoff, J. 2010. Chemical interactions between marine macroalgae and bacteria. *Mar Ecol Prog Ser*. **409**.
- Gromek, S., Suria, A., Fullmer, M., Garcia, J., Gogarten, J., Nyholm, S., Balunas, M. 2016. *Leisingera* sp. JC1, a Bacterial Isolate from Hawaiian Bobtail Squid Eggs, Produces Indigoidine and Differentially Inhibits Vibrios. *Front. Microbiol*. **7**: 1342.
- Jones, B., Nishiguchi, M. 2004. Counterillumination in the Hawaiian bobtail squid, *Euprymna scolopes* Berry (Mollusca: Cephalopoda). *Marine Biology*. **144**. 1151-1155.

- Kaplan, D., Cohen, Z., Abeliovich, A. 1985. Optimal Growth Conditions for *Isochrysis galbana*. *Biomass*. **9**: 39.
- Kerwin, A.H., S. Gromek, D.J. Deoss, K. O'Donnell, S. Frasca, M.J. Balunas, and S.V. Nyholm. 2017. The complex community of squid eggs: diverse functionality with a common goal. *In prep*.
- Kerwin, A. H. and Nyholm, S. V. 2017. Symbiotic bacteria associated with a bobtail squid reproductive system are detectable in the environment, and stable in the host and developing eggs. *Environ Microbiol*, **19**: 1463–1475. doi:10.1111/1462-2920.13665
- Lee, P.N., P. Callaerts, H.G. de Couet. 2009. The embryonic development of the Hawaiian Bobtail Squid (*Euprymna scolopes*). *Cold Spring Harbor Protocols*. **11**: doi:10.1101/pdb.ip77.
- McFall-Ngai, M.J. 2014. The importance of microbes in animal development: lessons from the squid-vibrio symbiosis. *Annual Review of Microbiology*. **68**: 177-194.
- Mieszkin, S., Callow, M., Callow, J. 2013. Interactions between microbial biofilms and marine fouling algae: a mini review. *Biofouling*, **29**: 1097-1113.
- Nyholm, S. V., and J. Graf. 2012. Knowing your friends: invertebrate innate immunity fosters beneficial bacterial symbioses. *Nature Reviews Microbiology*. **10**: 815-827.
- Singley, C. T. 1983. *Euprymna scolopes: Cephalopod Life Cycles*. London: Academic Press. **1**: 69-74.